Investigation of structure and enzymatic activity of the novel Rubisco from *Methanococcoides burtonii*

A Senior Honors Thesis

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by

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ABSTRACT

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the assimilation of carbon dioxide to organic matter. It is found in all domains of life, and is divided into four forms based on phylogenetics. The methanogenic archaeon *Methanococcoides burtonii* possesses a novel Rubisco whose sequence is intermediate between form II and form III. This enzyme is the first archaeal Rubisco shown *in vitro* to be aerotolerant. It also possesses a 26-residue "loop" structure that is unique among Rubisco proteins. It was found that deletion of this region significantly increases oxygen tolerance of the *M. burtonii* Rubisco *in vitro*. An *in vivo* expression system in *Rhodobacter capsulatus* was used to test the functionality of *M. burtonii* Rubisco. It was found that the Rubisco from *M. burtonii* is capable of physiologically significant carbon dioxide fixation. A mutant lacking the loop region was also functional *in vivo*, indicating that the expression system may be used in further studies concerning this novel structure.

BACKGROUND

The enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the most abundant protein found on earth (5). It catalyzes the carbon dioxide fixation reaction in the Calvin-Benson-Bassham (CBB) cycle, and is therefore of considerable environmental significance. Rubisco is found in all three kingdoms of life, and is classified based on sequence homologies into forms I, II, III, and IV. Forms I, II, and III catalyze bona fide Rubisco activity, while form IV Rubiscos, also known as the Rubisco-

like proteins (RLPs), do not. Form I is found in plants, algae, cyanobacteria, and many proteobacteria, while form II is found in proteobacteria and in one group of eukaryotes, the dinoflagellates. These two forms are the most well studied and are found in autotrophic organisms containing a full CBB pathway. Form III Rubisco is found only in archaea, and exists in organisms that do not have a complete CBB cycle and in many cases do not use CO_2 as a primary carbon source (6). They therefore do not seem to function in the same physiological role as form I and II Rubisco. It has been proposed that these Rubiscos function in an AMP recycling pathway (4) (Figure 1B).

In autotrophic organisms, Rubisco catalyzes the carboxylation of ribulose 1,5-bisphosphate (RuBP) to produce two molecules of 3-phosphoglycerate. However, low substrate specificity of Rubisco for CO_2 results in a competing reaction in which O_2 rather than CO_2 may be fixed, yielding 1 molecule of 3-phosphoglycerate and 1 molecule of 2-phosphoglycolate, and ultimately resulting in carbon loss (5) (Figure 1A). Studies of the structure and function of archaeal Rubisco proteins provide an opportunity to shed light on Rubisco evolution, as well as a key question in Rubisco biochemistry, the basis of CO_2/O_2 specificity. Many archaeal Rubisco enzymes are found in obligate anaerobes that have evolved in the absence of molecular oxygen. Interestingly, those archaeal Rubisco enzymes that have been studied were shown to be extremely oxygen-sensitive (1).

Methanococcoides burtonii is a psychrotolerant methanogen and an obligate anaerobe. Analyis of the derived primary amino acid sequence of the *M. burtonii* Rubisco (MBR) shows that this enzyme is unique in that its sequence places its identity between forms II and III (7) (Figure 2). MBR is most closely related to the form II proteins from

Rhodobacter capsulatus and *Rhodospirillum rubrum*, with 40% amino acid identity, and is 36% identical to its closest form III relative, the *Methanosarcina acetivorans* Rubisco (blastp, www.ncbi.nlm.nih.gov/blast/Blast.cgi). Like other archaea possessing Rubisco, *M. burtonii* does not contain the enzymes necessary for a complete CBB cycle. However, AMP phosphorylase (DeoA) and ribose 1, 5-bisphosphate isomerase (E2b2), the two enzymes besides Rubisco essential to the AMP-recycling pathway mentioned above, are present in *M. burtonii* (4). The most likely physiological role of MBR is therefore, like other form III Rubiscos, in this recycling pathway and not in carbon fixation.

Certain highly conserved amino acid residues indicate that MBR may be biochemically closer to form II than form III Rubisco in terms of CO_2/O_2 specificity. The active site "Rubisco motif" in MBR is KNDE, as in form II, rather than KDDE as in forms I and III. In addition, residue 306 of MBR is a valine, as in many form II enzymes, while form III enzymes have a conserved methionine at the analogous position. This conserved methionine in the form III Rubisco of *Archaeoglobus fulgidus* has been shown to be an important residue relative to oxygen sensitivity (2). Serine 363 in *A. fulgidus* was also found to be important in oxygen sensitivity, with an S363V mutant showing increased retention of activity in the presence of oxygen. The analogous residue is a conserved alanine in form I, isoleucine in form II, and serine or alanine in form III. In MBR, the analogous residue is a valine at position 398.

Another intriguing property of this enzyme is the presence of a 26-residue loop that has no homology to any known Rubisco, or to any protein in the Entrez database. As protein modeling indicates that this loop is distant from the active site, it was thought that these residues may be involved in structure but not directly in enzyme activity (7) (Figure

3A). One possible function of this loop is interaction with other enzymes. This study examined the effect of deletion of this loop to determine its importance in catalysis.

This study utilized *Rhodobacter capsulatus* SBI/II$^-$, a previously constructed Rubisco deletion strain of the photosynthetic bacterium *R. capsulatus* (3). *R. capsulatus* SBI/II$^-$ is incapable of growth under autotrophic conditions, and can therefore be used to examine the ability of a specific Rubisco or Rubisco mutant protein to support photoautotrophic growth under various conditions of CO_2 concentration and O_2 exposure (1). Complementation to photoautotrophic growth using archaeal Rubisco has previously been accomplished under conditions of strict anaerobiosis in a 20% CO_2/80% H_2 atmosphere using the *rbcL* gene from the mesophilic methanogen *Methanosarcina acetivorans* (5).

MATERIALS AND METHODS

Strains, Plasmids and Growth Conditions. *R. capsulatus* SBI/II$^-$ cultures for the mating procedure and starter cultures for photoautotrophic growth were grown aerobically in Peptone Yeast Extract (PYE) liquid media (3 g/l peptone, 3 g/l yeast extract, 10% Ormerod's basal salts, 1 µg/ml thiamine and niacin, and 0.1 µg/ml biotin) for 48 hours at 30º C with shaking. Routine maintenance of cultures and isolation of SBI/II$^-$ following mating was performed using PYE plates incubated at 30º C. When appropriate, antibiotics were added to the media at concentrations of 2.5 µg/ml tetracycline, 25 µg/ml spectinomycin, 25 µg/ml kanamycin, and 100 µg/ml rifampicin. Photoautotrophic cultures were grown in liquid Ormerod's Minimal Media (OMM) (10%

Ormerod's basal salts, 20% $(NH_4)_2SO_4$, 1 µg/ml thiamine and niacin, 0.1 µg/ml biotin, and 50 ml/l phosphate buffer) at 30º C with a constant light source. Anaerobiosis, the gas atmosphere employed, and the generation of growth curves are described below.

E. coli strains for genetic procedures and mating were grown in Luria-Bertani (LB) broth (10 g/l tryptone, 5 g/l yeast extract, 10 g/l sodium chloride, pH adjusted to 7.5) or on LB plates for 24 hours at 37º C. Antibiotics were used when appropriate at concentrations of 25 µg/ml kanamycin or 5 µg/ml tetracycline.

The pRPS-MCS3 plasmid was used for expression of Rubisco genes in *R. capulatus* SBI/II$^-$ under photoautotrophic conditions (5). It contains the *R. rubrum cbbM* promoter region, the gene for the transcriptional activator CbbR, and a tetracycline resistance gene (Figure 4).

Generation of deletion mutants. The wild-type *rbcL* gene from *M. burtonii* had previously been cloned into the plasmid pRPS-MCS3. To create specific deletions in the MBR gene, primers were designed using sequence flanking the regions to be deleted. The primer set MburMAXF (5'GCCATGAAGAAGTGCTGCCCAATCG3') and MburMAXR (5'TGACCATGTCTGCTCAAAGAAG3') were used to create the mutant dMax, which lacks the entire loop sequence. MburMidF (5'GATAGCTGGAGAGCCATGAAG3') and MburMidR (5'GGTATCCATTATCTTTGACCATGTCTG3') were used to create the dMid mutant, which lacks 20 amino acid residues from the middle of the loop region (Figure 3B). A PCR reaction containing Phusion Polymerase (New England Biolabs) and wild type *rbcL* in pRPS-MCS3 as a template was used to amplify a linear DNA fragment consisting of

the pRPS-MCS3 vector flanked by the segments of the *rbcL* gene terminating in either primer sequence.

The PCR products were digested with DpnI (New England Biolabs) to remove template DNA, and the 5' ends were phosphorylated using T4 Polynucleotide Kinase (New England Biolabs). The products were then circularized using T4 DNA Ligase (Invitrogen) and transformed into *E. coli* Top10 cells (Invitrogen) by electroporation. Plasmids were isolated from *E. coli* using a GeneJET Plasmid Miniprep Kit (Fermentas) and the genes were sequenced to confirm that the deletions were made and that no other mutations were introduced in the coding region.

Mating procedure. Triparental matings were performed using *E. coli* carrying wild type and mutant Rubisco genes in pRPS-MCS3, *R. capsulatus* SBI/II$^-$, and the helper strain *E. coli* 2013. Cells were washed with PYE and the mating mixture was incubated for 24 hours at 30 C on non-selective PYE plates. Cells were then streaked onto selective PYE plates containing tetracycline and rifampicin to isolate *R. capsulatus* cells that had taken up the pRPS-MCS3 plasmid.

Generation of growth curves. Transconjugants from the mating procedure were grown in selective PYE media, washed with OMM, and used to inoculate liquid photoautotrophic cultures in triplicate. Complementation to photoautotrophic growth using the wild-type *rbcL* gene was first attempted in jars of OMM bubbled with 5% CO_2/balance H_2. No steps were taken to remove oxygen from the media prior to inoculation. The form II Rubisco from *Rhodospirillum rubrum* has previously been shown to complement growth of strain SBI/II$^-$ under these conditions (5), so SBI/II$^-$ carrying *R. rubrum cbbM* in pRPS-MCS3 was used as a positive control. SBI/II$^-$ carrying

M. acetivorans rbcL was used as a negative control, as the *M. acetivorans* Rubisco has been shown to be incapable of supporting photoautotrophic growth under these conditions (1). 1-mL samples were removed at two to four day intervals using a syringe, and growth was monitored by measuring the absorbance at 660 nm.

In subsequent trials, growth curves were generated under stricter conditions of anaerobiosis. Media was placed in an anaerobic hood for several days prior to inoculation to allow for removal of oxygen. Cultures were grown in sealed, crimped tubes in an atmosphere of 20% CO_2 /balance H_2, with gases exchanged every 48 hours. *R. capsulatus* SBI/II$^-$ containing *R. rubrum cbbM* and *M. acetivorans rbcL* were again used as controls. The *M. acetivorans* Rubisco has been shown to be capable of complementing strain SBI/II$^-$ to photoautotrophic growth under strictly anaerobic conditions (1) and was therefore used to control for anaerobiosis. Unmated SBI/II$^-$ was used as a negative control. Growth curves for SBI/II$^-$ carrying the MBR loop deletion mutant dMax as well as wild-type MBR were generated under these conditions. Absorbance at 660 nm was measured at 24-hour intervals.

Once a culture ceased to grow, as indicated by an absorbance reading that was the same or lower than the previous reading, it was opened anaerobically for analysis. If the absorbance was above the accurate range of the spectrophotometer (1.0), a sample of the culture was diluted and the absorbance at 660 nm measured in order to determine the maximum absorbance. Samples of approximately 1-ml each were anaerobically transferred to airtight tubes, following which the cells were pelleted and the liquid media removed anaerobically. The cell pellets were flash frozen and stored at –80 C, for later analysis of Rubisco activity.

RESULTS AND DISCUSSION

Optimization of genetic procedures. Construction of deletion mutants was initially performed in the expression vector pSKB3, using the primers and procedure described in Materials and Methods. However, attempts to clone these mutants from pSKB3 into pRPS-MCS3 were unsuccessful. In the original cloning procedure, PCR was performed using the gene of interest in the pSKB3 vector as a template. Genes in pSKB3 were preceded by a sequence coding for a histidine tag. A primer was therefore designed to anneal to a 15-bp sequence at the start of the *rbcL* gene itself, to exclude the histidine tag. The primer also contained a 35-bp overhang coding for a ribosome binding site and a SpeI restriction site. The second primer was designed to anneal to the sequence following the stop codon, but also included a short 5' overhang containing a SacI restriction site. The restriction sites were introduced to allow for directional cloning into pRPS-MCS3.

Blunt PCR products were cloned into a TOPO Zero Blunt pCR vector (Invitrogen) and sequenced, and cloning into pRPS-MCS3 using the restriction sites SacI and SpeI was attempted. Digestions and purification of the linear DNA fragment from the TOPO vector repeatedly appeared successful, based on analysis by agarose gel electrophoresis. However, ligation and transformation consistently failed. A closer examination of the sequencing results revealed that two point mutations had been introduced during the PCR reaction. The first of these removed the gene's stop codon, and the second removed the SacI site that had been introduced in the primer sequence. This was not detected by gel electrophoresis because the TOPO vector contains a SpeI

restriction site flanking the coding region, so SpeI/SacI restriction digests yielded DNA fragments of the expected length.

The complications arising from this procedure were resolved by performing an additional initial PCR reaction with pSKB3 as a template, using the T7P and T7T primers to amplify a linear 2-kb DNA fragment containing the gene of interest. Using this linear fragment, in turn, as a template for PCR with the primers described above resulted in the desired product without additional mutations. This was confirmed by sequencing following insertion of the PCR products into the TOPO vector. However, by this time the mutations dMax and dMid had already been successfully created in pRPS-MCS3, as described in Materials and Methods. This method was therefore not pursued further in this study.

While the dMax and dMid deletions were created in pRPS-MCS3 as described, construction of the dMin mutant in pRPS-MCS3 was unsuccessful. It is likely that optimizing the PCR conditions or redesigning the primers to reduce mispriming would allow for the successful construction of dMin by this method. However, such modifications were not possible in the time frame of this study.

Rubisco activity assays by Brian Witte. Initial results of *in vitro* studies using recombinant MBR have yielded a number of revelations regarding the biochemical properties of the enzyme. Radiometric Rubisco activity assays were performed using lysate from *E. coli* cells in which expression of *rbcL* from an *E. coli* expression vector had been induced using IPTG. All archaeal Rubiscos described so far are extremely oxygen sensitive, losing all or nearly all of their carboxylation activity in the presence of oxygen (1, 2). However, MBR retained approximately 30-35% activity in the presence of

oxygen (Table 1), making it the first described aerotolerant methanogen Rubisco. In contrast to *M. acetivorans* Rubisco (1), it regained full activity in anaerobic conditions following exposure to oxygen. Oxygen sensitivity of MBR, like its sequence, seems to be intermediate between forms II and III. Studies using purified recombinant MBR, and especially the determination of its kinetic properties, would allow for more rigorous comparison with other known Rubisco and likely provide further insights into its biochemical characteristics.

The loop deletion mutants described above (Figure 3B) were also expressed in *E. coli* and the lysate assayed for Rubisco activity. Conclusions concerning anaerobic activity of each of these mutants compared to the wild-type will have to be drawn based on assays of purified protein, which have not yet been performed. However, this preliminary evidence indicates that the dMid mutant has much less activity compared to the wild-type as well as the other deletion mutants. It is possible that deletion of a portion of the loop as in dMid results in disruption of the enzyme's structure due to interaction of the remaining residues with other parts of the enzyme. The deletion of the entire loop also results in a significant decrease in activity, although not to the extent of the partial deletion. The 2-residue deletion mutant dMin showed anaerobic activity that approaches that of wild-type MBR, which may indicate that this small deletion did not disrupt the overall structure of the enzyme.

Most interestingly, the results of several trials indicate that all three deletion mutants tolerate oxygen much better than the wild-type MBR, becoming comparable in this respect to form II enzymes. While wild type MBR lost 65 to 71% of its activity in the presence of oxygen, the deletion mutants dMax, dMid, and dMin lost only 31, 24 and

37% of their anaerobic activities, respectively (Table 1B). Given the distance of the loop from the active site, this effect on the catalytic properties of the enzyme was unexpected. Further studies with purified recombinant proteins will be necessary to elucidate the role of the loop sequence in determining the biochemical properties of MBR.

Complementation of *R. capsulatus* SBI/II$^-$ with *M. burtonii rbcL* and mutants. Complementation of *R. capsulatus* SBI/II$^-$ with the wild-type MBR gene was initially attempted under conditions of anaerobiosis and CO_2 concentration sufficient for growth using form II Rubiscos, as described in Materials and Methods. In the past, it has been shown that these conditions do not provide sufficient exclusion of oxygen to allow for autotrophic growth with the form III enzyme from *Methanosarcina acetivorans*. *M. acetivorans* Rubisco is completely and irreversibly inhibited in the presence of oxygen (1). It was found in this study that despite its similarities to form II Rubiscos, MBR was unable to complement growth under conditions amenable to growth with form II enzymes.

Stricter conditions of anaerobiosis, described in Materials and Methods, were subsequently employed in an attempt at complementation to photoautotrophic growth using wild-type MBR and the dMax deletion mutant. The exclusion of the dMin mutant from this portion of the study is addressed above. The dMid mutant was not included because, though it was successfully constructed and mated into *R. capsulatus* SBI/II$^-$, the transconjugant failed to grow in PYE liquid culture to a density comparable to the other starter cultures for photoautotrophic growth. At the time the starter cultures were inoculated, growth was barely apparent on the PYE plate containing the dMid transconjugants used as the inoculum. A second attempt following more substantial

growth of the plated transconjugants would likely have yielded a sufficient starter culture, but time restrictions did not allow for this to be done.

R. capsulatus SBI/II$^-$ cultures grew photoautotrophically under anaerobic conditions when complemented with either wild-type *M. burtonii* RbcL or the dMax deletion mutant (Figure 5). The three controls performed as expected. *R. capsulatus* SBI/II$^-$ alone did not grow, indicating that there was no adaptive mutation in the strain itself or contamination of the cultures (Figure 5). Both *R. rubrum* CbbM-complemented and *M. acetivans* RbcL- complemented SBI/II$^-$ were able to grow (Figure 5). The former indicates that no additional factors hindered growth of the cultures, while the latter demonstrates that anaerobiosis was maintained.

Growth complemented with the form III Rubisco enzymes from *M. burtonii* and *M. acetivorans*, as well as the form II Rubisco from *R. rubrum*, reached comparable absorbance maxima of 1.5, 1.4, and 1.5, respectively (Table 2). However, for the *R. rubrum cbbM* -complemented culture, this maximum was reached after 12 days, while it took the *M. burtonii rbcL* -complemented culture 21 days and the *M. acetivorans rbcL*-complemented culture 23 days to reach this absorbance value (Table 2). The difference can be attributed to the longer lag time of cultures complemented with archaeal Rubisco (Figure 5), as well as their slightly longer doubling times (Table 2). These characteristics could be due to many factors, including difficulties in expression in the host organism (see below), or differences in levels of carboxylase activity between the Rubisco enzymes (Table 1). The cultures complemented with MBR dMax grew much slower than the others, with a doubling time of 9 days, compared to the second longest doubling time of 4 days for wild-type MBR (Table 2). At the time of this writing, these cultures have not

reached an absorbance maximum, but are approaching the density reached by the other cultures (Figure 6, Table 2). The slow growth could be due to low levels of anaerobic activity compared to wild-type MBR, as observed in recombinant Rubisco assays (Table 1), or to structural instability of the deletion mutant.

One possible hindrance to optimal growth of strain SBI/II$^-$ complemented with archaeal Rubisco is the prevalence of rare codons in archaeal genes. This is especially true in *M. burtonii rbcL* (Figure 6B). Gene expression studies in *E. coli* showed extremely low expression of the MBR gene in strains not containing a Rosetta plasmid (Novagen) to provide rare tRNAs (Figure 6A). In SBI/II$^-$, this is expected to be even more problematic due to the much greater codon bias seen in *R. capsulatus* (Figure 6B). Therefore, before further complementation studies using this gene can be performed, especially in more challenging conditions or using mutants with lower activity, it would be advisable to construct a plasmid containing rare tRNA genes for expression in *R. capsulatus*. Introduction of rare tRNAs into SBI/II$^-$ would make this *in vivo* expression system more effective for studying archaeal Rubiscos in general.

MBR-complemented growth of *R. capsulatus* SBI/II$^-$ demonstrates that MBR is a functional Rubisco, capable of physiologically significant RuBP-dependent CO_2 fixation. Despite its high oxygen tolerance relative to other archaeal Rubisco, it is only able to complement growth under the rigorous anaerobic conditions previously used for complementation with the highly oxygen sensitive Rubisco from *M. acetivorans*. It seems that the affinity of MBR for oxygen is still high enough that it will not support growth in the presence of small amounts of oxygen even with high concentrations of CO_2. In this sense, MBR behaves more like a form III Rubisco than form II. Further studies may test

the enzyme's affinity for CO_2 by attempting photoautotrophic growth with reduced CO_2 concentrations.

The ability of the dMax mutant to complement SBI/II$^-$ to photoautotrophic growth opens the possibility for further *in vivo* examination of the properties of MBR loop deletion mutants. If the other deletion mutants also complement growth under the conditions utilized here, complementation to photoautotrophic growth may be attempted with reduced CO_2 concentrations, as well as under microaerobic conditions to assess the ability of these mutants to distinguish between CO_2 and O_2. The mutant dMin, with anaerobic activity levels that approach the wild-type *in vitro*, is an especially attractive candidate for study in this system.

While this type of experiment can elucidate the effect of the loop region on biochemical properties of MBR, it cannot reveal why this region exists in the *M. burtonii* enzyme alone among all Rubisco known to date. Discovery of a physiological function for this region would require study in its native organism. Understanding how this region fits into the overall structure of the enzyme would likely be revealing as well, but as lack of homology limits the utility of *in silico* modeling, this would require determination of an X-ray crystal structure for MBR.

Further studies with MBR will likely provide a more complete understanding of this novel enzyme. Its unique properties offer an opportunity to gain insight into structure-function relationships in Rubisco, as well as into Rubisco evolution.

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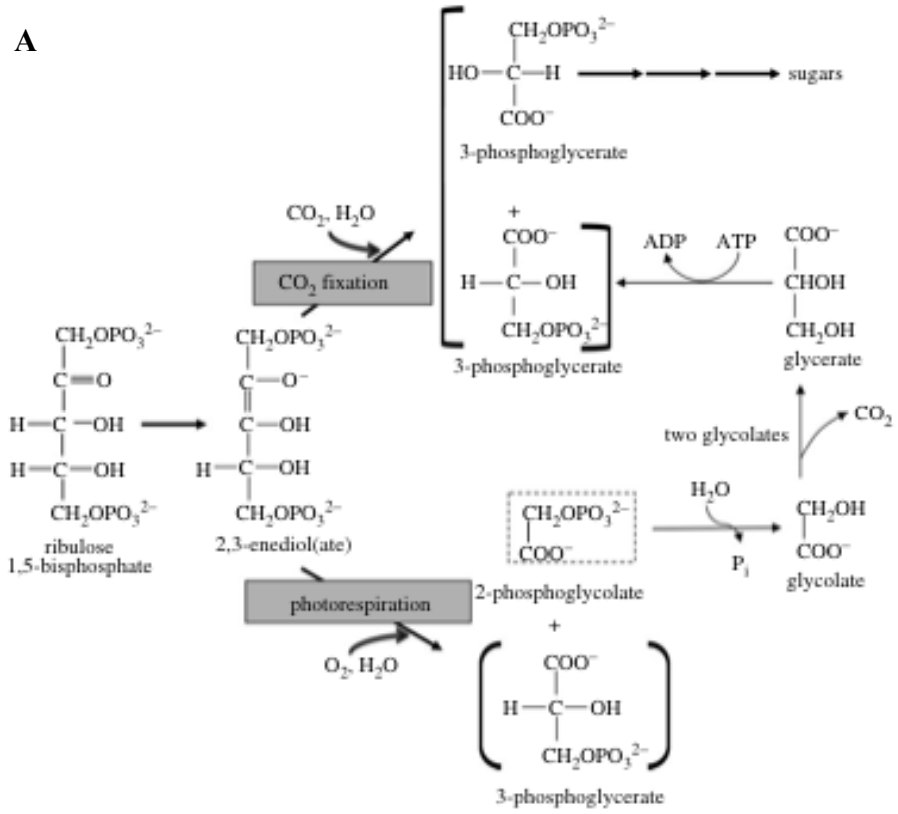
Figure 1. Catalytic activity of ribulose 1,5-bisphosphate carboxylase/oxygenase. (A) The carboxylase and oxygenase reactions catalyzed by Rubisco (7). (B) Physiological role of Rubisco in archaea as part of an AMP salvage pathway. DeoA catalyzes the phosphorylation of adenosine 5'-monophosphate (AMP) to produce ribose 1,5-bisphosphate (R15P) and release the adenine base. E2b2 acts as a R15P isomerase, producing the Rubisco substrate ribulose 1,5-bisphosphate. This is carboxylated by Rubisco and the 3-phosphoglycerate products are diverted into central carbon metabolism. DeoA and E2b2 activities have been confirmed for the enzymes from *Thermococcus kodakaerensis*, and homologs of these are known to exist in *M. burtonii* (4).

A


3-phosphoglycerate
sugars
CO_2, H_2O
CO_2 fixation
ADP
ATP
glycerate
ribulose
1,5-bisphosphate
2,3-enediol(ate)
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photorespiration
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B

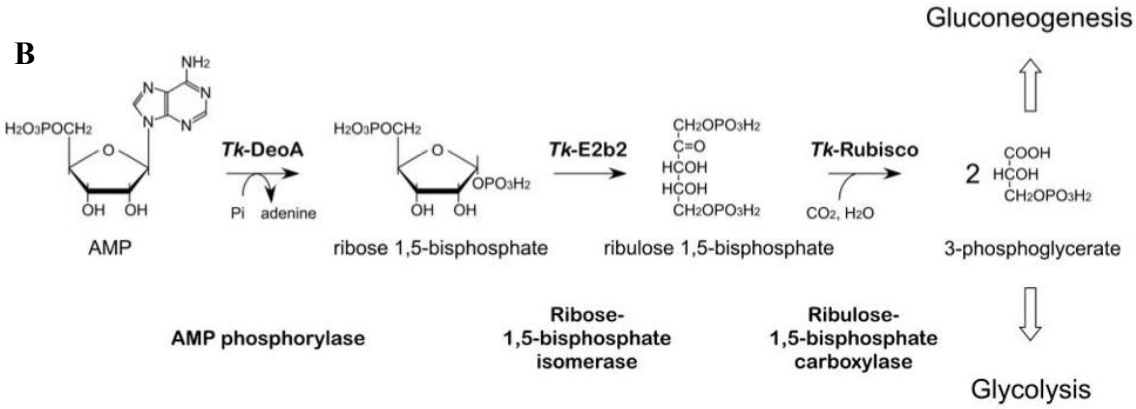

Gluconeogenesis
Tk-DeoA
Pi
adenine
Tk-E2b2
Tk-Rubisco
CO_2, H_2O
AMP
ribose 1,5-bisphosphate
ribulose 1,5-bisphosphate
3-phosphoglycerate
AMP phosphorylase
Ribose-
1,5-bisphosphate
isomerase
Ribulose-
1,5-bisphosphate
carboxylase
Glycolysis

Figure 2. Rubisco phylogenetic tree. Branches for forms I, II, and III are circled and labeled. RLP, Rubisco-like proteins, are form IV. The *M. burtonii* Rubisco is circled in yellow (Brian Witte, personal communication).

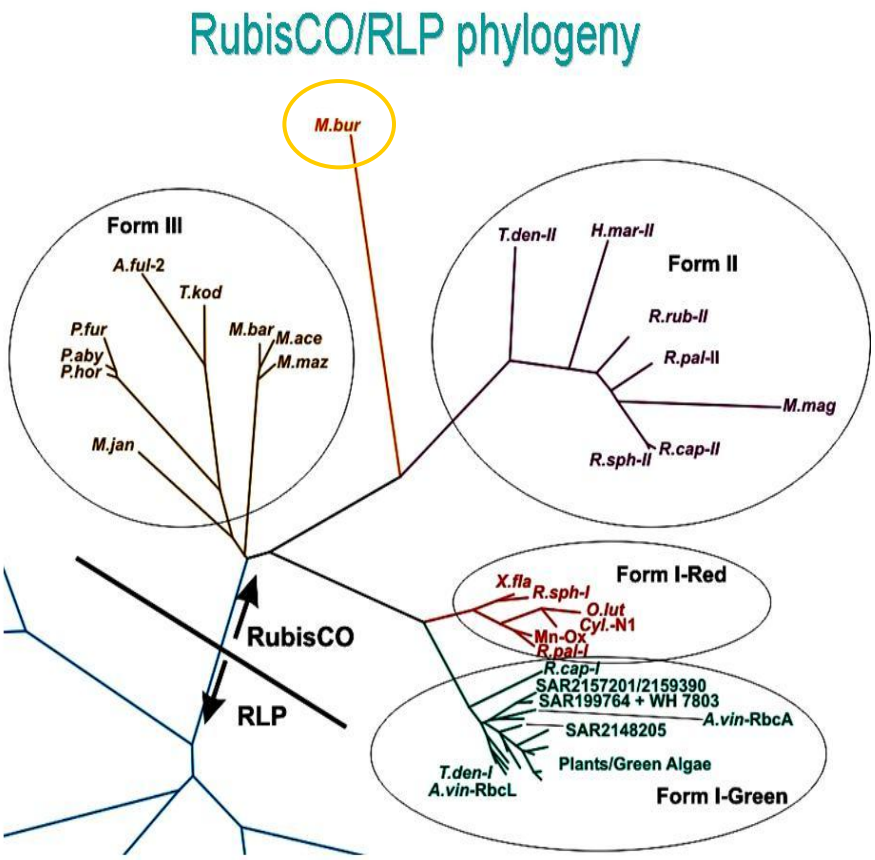
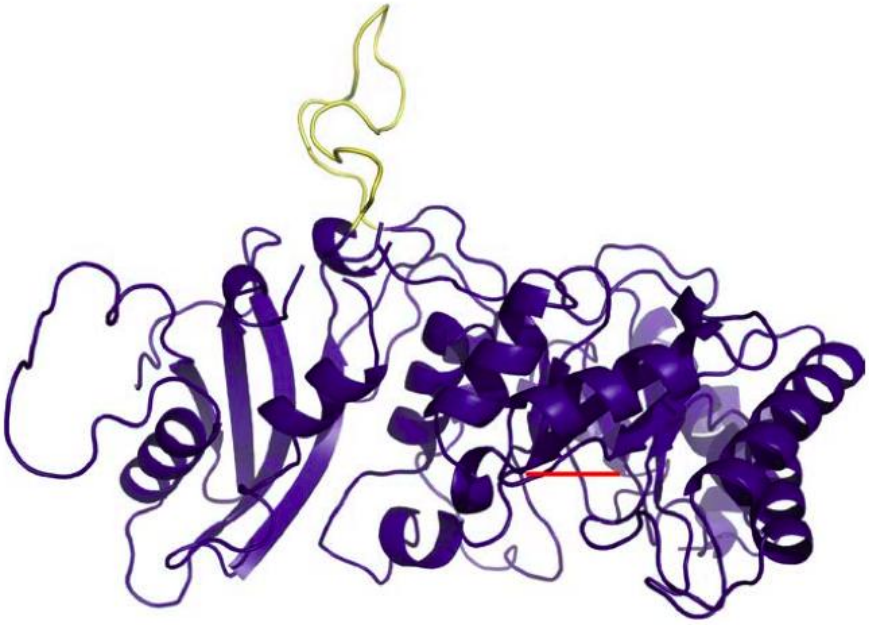

RubisCO/RLP phylogeny
M.bur
Form III
A.ful-2
T.kod
P.fur
P.aby
P.hor
M.bar
M.ace
M.maz
M.jan
Form II
T.den-II
H.mar-II
R.rub-II
R.pal-II
M.mag
R.sph-II
R.cap-II
Form I-Red
X.fla
R.sph-I
O.lut
Cyl.-N1
Mn-Ox
R.pal-I
R.cap-I
SAR2157201/2159390
SAR199764 + WH 7803
A.vin-RbcA
SAR2148205
Plants/Green Algae
T.den-I
A.vin-RbcL
Form I-Green
RubisCO
RLP

Figure 3. The *M. burtonii* Rubisco loop. (A) Protein model showing loop in yellow (7). (B) Protein sequence alignment with *R. capsulatus* (form II) and *M. acetivorans* (form III) Rubiscos. The *M. burtonii* loop sequence is shown in red, and is equivalent to the sequence deleted in the mutant dMax. The missing residues in the dMid mutant are highlighted in blue, and those removed in the dMin mutant are underlined. (Alignments from ClustalW2, www.ebi.ac.uk/Tools/ clustalw2/index.html)

A



B

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R.capsulatus_Form_II    DKIMAYMLNDEAAQG--------PFYHQDWL----------------------------GMKATT
M.acetivorans_Form_III DKDEVLSIRDECVLDTVPADPEQHVLAQDWG----------------------------GLKPMF
M.burtonii              DVVAAHSIQYLKSPG--------HFFEQTWSKIMDTDKDVINLVNEDLAHHVILEDDSWRAMKCC
                        *   .  :.    .         .  * *                          :*
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dMin

dMid

dMax

Figure 4. The pRPS-MCS3 expression plasmid. The broad host-range plasmid contains the *cbbM* promoter from *R. rubrum*, pcbbM, the gene for its transcriptional activator, *cbbR*, and a multiple cloning site. The mobilization site (Mob) enables transfer of the plasmid to *R. capsulatus*, and the tetracycline resistance gene (Tet) allows for selection (5).

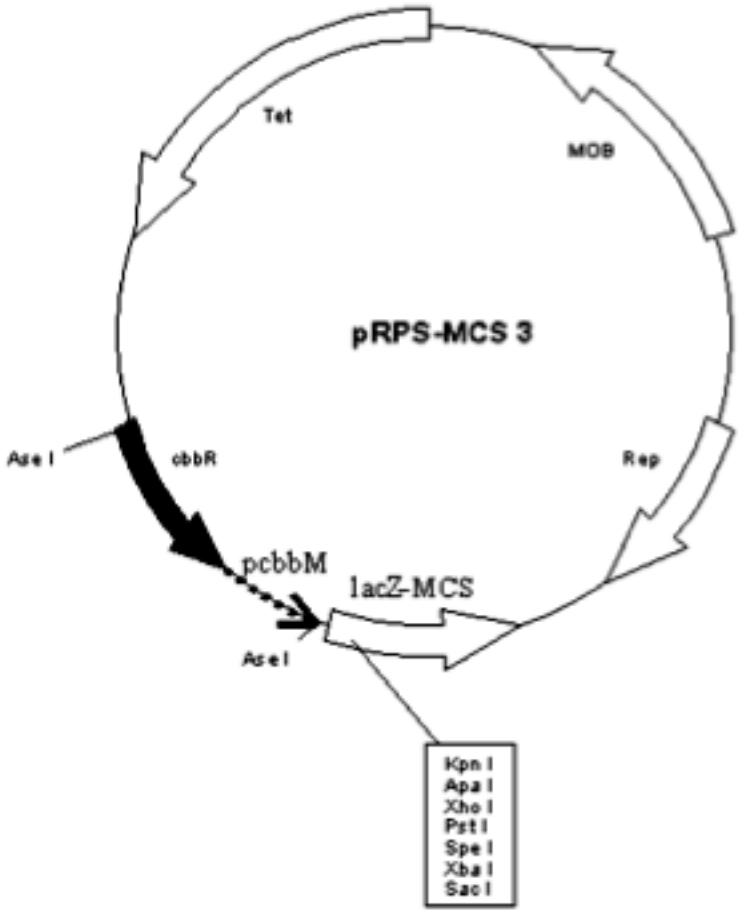

Tet
MOB
pRPS-MCS 3
Ase I
cbbR
Rep
pcbbM
lacZ-MCS
Ase I
Kpn I
Apa I
Xho I
Pst I
Spe I
Xba I
Sac I

Table 1. Rubisco activity assays using *E. coli* cell lysates. Specific activities (S.A.) are given in nanomoles of CO_2 fixed per minute per milligram of protein. Change in activity is given as percentage of anaerobic activity lost in the presence of O_2 (Brian Witte, personal communication). (A) Wild-type MBR and the form II Rubisco from *R. rubrum*. (B) Wild-type MBR and MBR deletion mutants dMax, dMid, and dMin.

A

Activity of MBR and form II Rubisco			
	Anaerobic	Aerobic	% Change in S.A.*
R. rubrum CbbM	781	464	41
MBR (wild-type)	393	115	71

B

Activity of MBR wild-type and deletion mutants			
	Anaerobic	Aerobic	% Change in S.A.*
Wild-type	272	97	65
dMax	33	23	31
dMid	10	8	24
dMin	200.2	125.6	37

*%Change= [(Anaerobic S.A.-aerobic S.A.)/anaerobic S.A.] x 100

Figure 5. Photoautotrophic growth of *R. capsulatus* strain SBI/II$^-$ containing *R. rubrum cbbM* (×), *M. acetivorans rbcL* (■), *M. burtonii rbcL* (●), and *M. burtonii rbcL* dMax (♦) in plasmid pRPS-MCS3. ▲, *R. capsulatus* SBI/II$^-$ without pRPS-MCS3. Cultures were grown in sealed, crimped tubes with an atmosphere of 20% CO_2 balanced with H_2, and gases were exchanged every 48 hours.

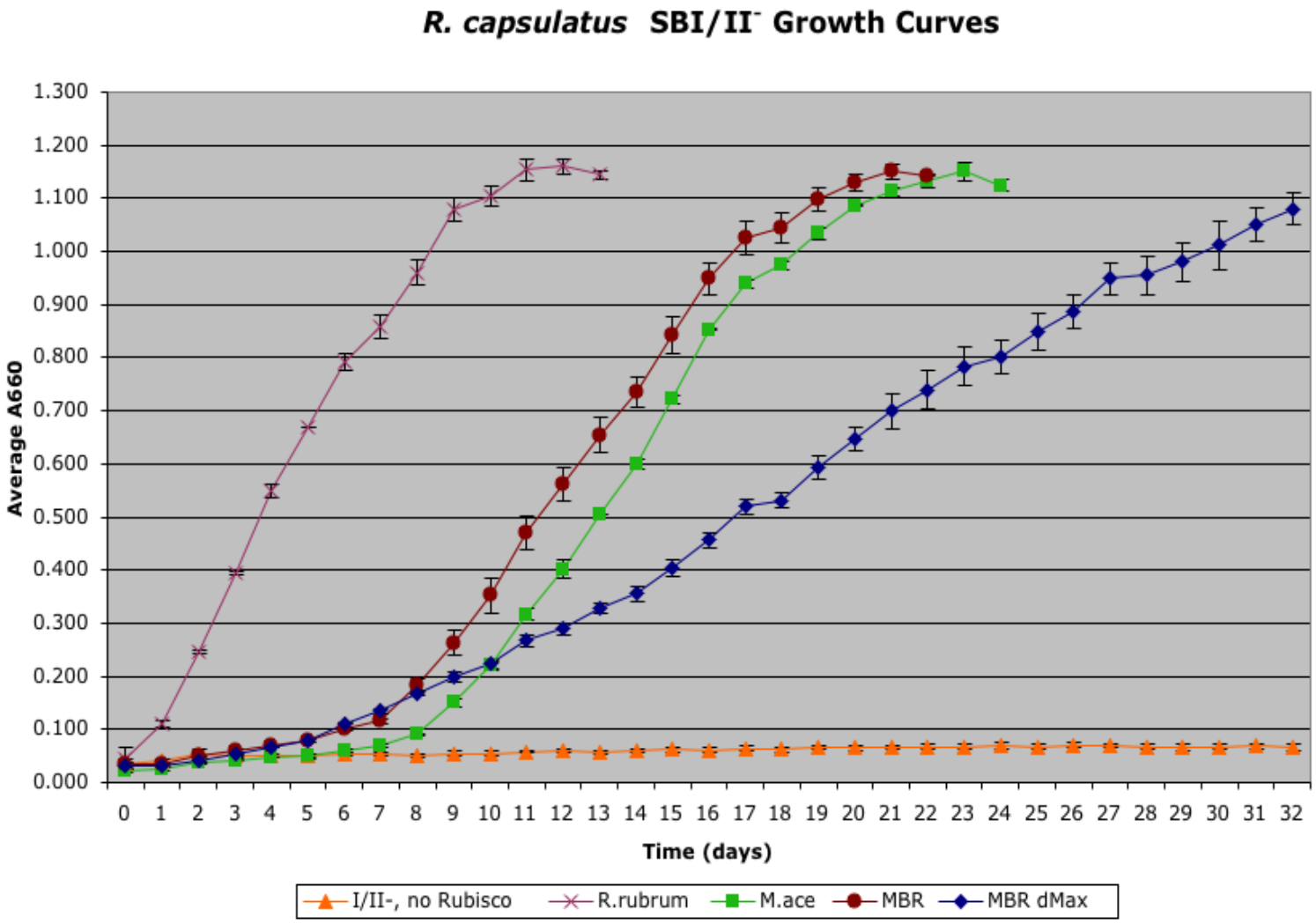

R. capsulatus SBI/II- Growth Curves
Average A660
1.300
1.200
1.100
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
Time (days)
I/II-, no Rubisco
R.rubrum
M.ace
MBR
MBR dMax

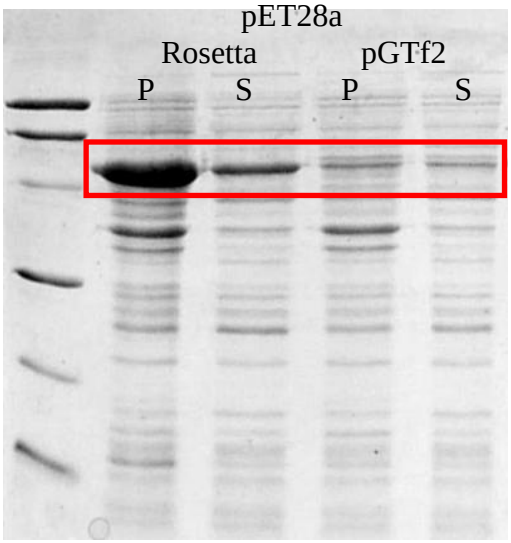
Table 2. Summary of growth curve results. Absorbance maxima were calculated from absorbance values obtained using two-fold dilutions of the final cultures, as absorbance values above 1 are beyond the accurate range of the spectrophotometer. The doubling time was estimated from the graph of the growth curve (Figure 5), and reflects the time interval over which the cultures grew from an A_{660} of 0.4 to 0.8.

Rubisco	Maximum A_{660} (average)	Time to reach maximum A_{660} (days)	Approximate doubling time (days)
MBR	1.5	21	4
MBR dMax	>1*	>32*	9
R. rubrum CbbM	1.5	12	3
M.acetivorans RbcL	1.4	23	3.5

*Final data not available as cultures were still growing at the conclusion of this study.

Figure 6. Rare codon usage and recombinant protein accumulation. (A) Cell lysate of IPTG-induced *E. coli* carrying the MBR gene in the expression vector pet28a (Novagen). The Rosetta plasmid (Novagen) supplies tRNAs for rare codons, and pGTf2 supplies chaperones GroEL, GroES, and proline isomerase. The 52.8 kDa band corresponding to MBR is outlined in red. P, pellet fraction; S, soluble fraction (Brian Witte, personal communication). (B) Codon usage chart showing frequency per 1000 codons of selected codons in *E. coli* and *R. capsulatus* alongside the number of occurrences of these codons in the *M. burtonii* and *M. acetivorans rbcL* genes. Codons for which tRNAs are supplied by the Rosetta plasmid are marked with an asterisk. Codon frequencies under 2.5 are shown in red. Bold marks occurrences of codons in the *M. burtonii* gene that do not occur in the *M. acetivorans* gene. (Data from Kazusa DNA Research Institute, Codon Usage Database, www.kazusa.or.jp/codon)

A



B

		Host organism Freq/1000		**Gene** Total number occurences	
Codon		*E.coli*	*R. capsulatus*	Mbur *rbcL*	Mace *rbcL*
Leu	UUA	17.5	0.3	**2.00**	**0.00**
	CUA*	3.4	0.3	1.00	2.00
Ser	UCU	8.5	1.4	0.00	1.00
	UCA	6.1	0.8	**12.00**	**0.00**
	AGU	9.0	1.2	2.00	3.00
Cys	UGU	4.2	1.4	2.00	1.00
Pro	CCU	5.8	1.9	3.00	7.00
	CCC*	2.4	20.1	4.00	5.00
	CCA	7.4	0.9	7.00	3.00
Ile	AUA*	5.0	0.4	**8.00**	**0.00**
Thr	ACU	7.7	1.3	3.00	4.00
	ACA	6.1	1.3	7.00	5.00
Arg	CGA*	2.4	1.5	**2.00**	**0.00**
	AGA*	2.4	0.6	**8.00**	**0.00**
	AGG*	2.1	1.6	4.00	8.00
Val	GUA	10.6	0.5	11.00	5.00
Gly	GGA*	8.2	2.9	16.00	9.00